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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 35 USC 111(b).

1. INVENTOR(s) APPLICANT(s)			
Last Name	First Name	Middle Initial	Residence
DAHLQUIST	Anders		Svalov, Sweden
STAHL	Ulf		Uppsala, Sweden
LENMAN	Marit		Svalov, Sweden
Banas	Antoni		Svalov, Sweden
Lee	Michael		
SANDAGER	Line		Savlov, Sweden
Ronne	Hans		Uppsala, Sweden
STYMNE	Sten		Svalov, Sweden

2. TITLE OF THE INVENTION: **PDAT:NOVEL ENZYME THAT CATALYZES THE ACYL-CoA-INDEPENDENT FORMATION OF TRIACYLGLYCEROL IN YEA ST AND PLANTS**

3. CORRESPONDENCE ADDRESS:

Keil & Weinkauf
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
(202) 659-0100; (202) 659-0105-fax

4. ENCLOSED APPLICATION PARTS:

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5. METHOD OF PAYMENT

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Respectfully submitted,
KEIL & WEINKAUF

Jason D. Voight

Jason D. Voight
Reg. No. 42,205

1101 Connecticut Ave., N.W.
Washington, D.C. 20036
(202)659-0100

JDV/lc

Classification: Biochemistry

PDAT: a novel enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants†

Keywords: (oil seeds / PDAT /phospholipid : diacylglycerol acyltransferase / phospholipids / triacylglycerol / yeast)

Anders Dahlqvist[1], Ulf Ståhl[2], Marit Lenman[1], Antoni Banas[1], Michael Lee, Line Sandager[1], Hans Ronne[2] and Sten Stymne[1]

[1]Department of Plant Breeding Research, Swedish University of Agricultural Sciences, Herman Ehles väg 2-4,S-268 31 Svalöv, Sweden

[2]Department of Plant Biology, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Box 7080, S-750 07 Uppsala, Sweden

†The first two authors made equal contributions to this work.

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ABSTRACT Triacylglycerol (TAG) is known to be synthesized in a reaction that uses acyl-CoA as acyl donor and diacylglycerol (DAG) as acceptor, a reaction which is catalyzed by the enzyme acyl-CoA: diacyiglycerol acyltransferase. We have found that some plants and yeast in addition to this reaction also have an acyl-CoA-independent mechanism for TAG synthesis. which uses phospholipids as acyl donors and DAG as acceptor. This reaction is catalyzed by a novel enzyme named by us as a phospholipid: diacylglycerol acyltransferase, or PDAT. PDAT was first characterized in microsomal preparations from three different oil seeds (sunflower, castor bean and *Crepis palaestina*). The specificity of the enzyme for the acyl group in the phospholipid varies between these species. Thus, *C. palaestina* microsomal membranes preferentially incorporate vernoloyl (12,13-epoxy-9oxtadecenoyl) groups into TAG whereas PDAT in microsomes of castor bean has preferences for both ricinoleoyl (12-hydroxy-octadecenoyl) and vernoloyl groups as compared to oleoyl groups. This suggests that PDAT could play a role in the removal of bilayer disturbing fatty acids from phospholipids in seeds where such fatty acids are abundant in the TAG. PDAT activity is also present in yeast microsomes. The substrate specificity of this PDAT is dependent on the headgroup of the acyl donor, the acyl group transferred, and the acyl chains of the acceptor DAG. The gene encoding the enzyme was identified as YNR008w. The PDAT protein is related to lecithin : cholesterol acyltransferase, which catalyzes the acyl-CoA independent synthesis of cholesterol esters. However, the budding yeast PDAT and its homologs in fission yeast and *Arabidopsis* form a distinct branch within this protein superfamily, indicating that a separate PDAT enzyme arose at an early point in evolution.

INTRODUCTION

Triacylglycerol (TAG) is the most common lipid-based energy reserve in nature. The main pathway for synthesis of TAG is believed to involve three sequential acyl-transfers from acyl-CoA to a glycerol backbone (1, 2). For many years, acyl-CoA : diacylglycerol acyltransferase (DAGAT), which catalyzes the third acyl transfer reaction, was thought to be the only unique enzyme involved in TAG synthesis. It acts by diverting diacylglycerol (DAG) from membrane lipid synthesis into TAG (2). Genes encoding this enzyme were recently identified both in the mouse (3) and in plants (4, 5), and the encoded proteins were shown to be homologous to acyl-CoA : cholesterol acyltransferase (ACAT). It was also recently reported that another DAGAT exists in the oleaginous fungus *Mortierella ramanniana*, which is unrelated to the mouse DAGAT, the ACAT gene family or to any other known gene (6). In addition to these acyl-CoA dependent enzymes, recent work has shown that in microsomal preparations from oil seeds, TAG synthesis can also occur in the absence of acyl-CoA (7, 8). However, the enzyme involved in this acyl-CoA-independent synthesis of TAG has not yet been identified in any organism.

In this paper, we characterize the acyl-CoA-independent synthesis of TAG in plants, and conclude that it is mediated by a novel enzyme which we call phospholipid : diacylglycerol acyltransferase (PDAT). The new enzyme is proposed to be involved in the accumulation of high levels of hydroxylated fatty acid (ricinoleic acid) and epoxidated fatty acid (vernolic acid) in TAG in castor bean (*Ricinus Communis)* and the hawk's-beard *Crepis palaestina*, respectively. Furthermore, a similar enzyme is shown to be present in the yeast *Saccharomyces cerevisiae*, and the gene encoding this enzyme, YNR008w, is identified.

MATERIALS AND METHODS

Yeast strains and plasmids. The wild type yeast strains used were either FY1679 (*MATα his3-Δ200 leu2-Δ1 trp1-Δ6 ura3-52*) or W303-1A (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) (10). The YNR008w::KanMX2

disruption strain FVKT004-04C(AL), which is congenic to FY1679, was obtained from the Euroscarf collection (9). A 2751 bp fragment containing the YNR008w gene with 583 bp of 5' and 183 bp of 3' flanking DNA was amplified from W303-1A genomic DNA using *Taq* polymerase with 5'-TCTCCATCTTCTGCAAAACCT-3' and 5'-CCTGTCAAAAACCTTCTCCTC-3' as primers. The resulting PCR product was purified by agarose gel electrophoresis and cloned into the *Eco*RV site of pBluescript. For complementation experiments, the cloned fragment was released from pBluescript by *Hin*dIII-*Sac*I digestion and then cloned between the *Hin*dIII and *Sac*I sites of pFL39 (11), thus generating pUS1. For overexpression of the PDAT gene, a 2202 bp *Eco*RI fragment from the pBluscript plasmid which contains only 24 bp of 5' flanking DNA was cloned into the BamHI site of the *GAL1-TPK2* expression vector pJN92 (12), thus generating pUS4.

Microsomal preparations. Microsomes from developing seeds of sunflower (*Helianthus annuus*), *Ricinus communis* and *Crepis palaestina* were prepared using the procedure of Stobart and Stymne (13). To obtain yeast microsomes, 1g of yeast cells (fresh weight) was re-suspended in 8 ml of ice-cold buffer (20 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5 % (v/v) glycerol, 1 mM DTT, 0.3 M ammonium sulfate) in a 12 ml glass tube. To this tube, 4 ml of glass beads (diameter 0.45-0.5 mm) were added, and the tube was then heavily shaken (3 x 60 s) in an MSK cell homogenizer (B. Braun Melsungen AG, Germany). The homogenized suspension was centrifuged at 20,000 x g for 15 min at 6°C and the resulting supernatant was again centrifuged at 100,000 x g for 2 h at 6°C. The 100,000 x g pellet was resuspended in 0.1 M potassium phosphate (pH 7.2), and stored at -80°C. It is subsequently referred to as the crude yeast microsomal fraction.

Lipid substrates. Radio-labeled ricinoleic (12-hydroxy-octadecenoic) and vernolic (12,13-epoxy-octadecenoic) acids were synthesized enzymatically from [1-^{14}C]oleic acid and [1-^{14}C]linoleic acid, respectively, by incubation with microsomal preparations from seeds of *Ricinus communis* and *Crepis palaestina*, respectively (14). The synthesis of phosphatidylcholines (PC) or phosphatidylethanolamines (PE) with ^{14}C-labeled acyl groups in the *sn*-2 position was performed using either enzymatic (15), or synthetic (16) acylation of [^{14}C]oleic, [^{14}C]ricinoleic, or [^{14}C]vernolic acid. Dioleoyl-PC that was labeled in the *sn*-1 position was synthesized from *sn*-1-[^{14}C]oleoyl-lyso-PC and unlabeled oleic acid as described in (16). *Sn*-1-

oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG was synthesized from PC by the action of phospholipase C type XI from *B. Cereus* (Sigma Chemical Co.) as described in (17). Monovernoloyl- and Divernoleoyl-DAG were synthesized from TAG extracted from seeds of *Euphorbia lagascae*, using the TAG-lipase (Rizhopus arrhizus, Sigma Chemical Co.) as previously described (7). Monoricinoleoyl-TAG was synthesized according to the same method using TAG extracted from Castor bean.

Lipid analysis. Total lipid composition of yeast were determined from cells harvested from a 40 ml liquid culture, broken in a glass-bead shaker and extracted into chloroform as described by Bligh and Dyer (18), and then separated by thin layer chromatography in hexane/diethylether/acetic acid (80:20:1) using pre-coated silica gel 60 plates (Merck). The lipid areas were located by brief exposure to I_2 vapors and identified by means of appropriate standards. Polar lipids, sterol-esters and triacylglycerols, as well as the remaining minor lipid classes, referred to as other lipids, were excised from the plates. Fatty acid methylesters were prepared by heating the dry excised material at 85 °C for 60 min in 2% (v/v) sulfuric acid in dry methanol. The methyl esters were extracted with hexane and analyzed by GLC through a 50 m x 0.32 mm CP-Wax58-CB fused-silica column (Chrompack), with methylheptadecanoic acid as an internal standard. The fatty acid content of each fraction was quantified and used to calculate the relative amount of each lipid class. In order to determine the total lipid content, 3 ml aliquots from yeast cultures were harvested by centrifugation and the resulting pellets were washed with distilled water and lyophilized. The weight of the dried cells was determined and the fatty acid content was quantified by GLC-analyses after conversion to methylesters as described above. The lipid content was then calculated as nmol fatty acid (FA) per mg dry weight yeast.

Enzyme assays. Aliquots of crude microsomal fractions (corresponding to 10 nmol of microsomal PC) from developing plant seeds or yeast cells were lyophilized over night. ^{14}C-Labeled substrate lipids dissolved in benzene were then added to the dried microsomes. The benzene was evaporated under a stream of N_2, leaving the lipids in direct contact with the membranes, and 0.1 ml of 50 mM potassium phosphate (pH 7.2) was added. The suspension was thoroughly mixed and incubated at 30°C for the time period indicated, up to 90 min. Lipids were extracted from the reaction mixture using chloroform and separated by thin layer chromatography in hexane/diethylether/acetic acid (35:70:1.5) using silica gel 60 plates (Merck). The

radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager, Packard, US).

Yeast cultivation. Yeast cells were grown at 28°C on a rotatory shaker in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose), synthetic medium (19) containing 2% (v/v) glycerol and 2% (v/v) ethanol, or minimal medium (20) containing 16 g/l of glycerol.

RESULTS

Acyl-CoA-independent synthesis of TAG by oil seed microsomes. A large number of unusual fatty acids can be found in oil seeds (21). Many of these fatty acids, such as ricinoleic (22) and vernolic acids (23), are synthesized using PC with oleoyl or linoleoyl groups esterified to the *sn*-2 position, respectively, as the immediate precursor. However, even though PC can be a substrate for unusual fatty acid synthesis and is the major membrane lipids in seeds, unusual fatty acids are rarely found in the membranes. Instead, they are mainly incorporated into the TAG. A mechanism for efficient and selective transfer of these unusual acyl groups from PC into TAG must therefore exist in oil seeds accumulating those. This transfer reaction was biochemically characterized in seeds from castor bean (*Ricinus communis)* and *Crepis palaestina*, plants which accumulate high levels of ricinoleic and vernolic acid, respectively, and sunflower (*Helianthus annuus)*, a plant which has only common fatty acids in its seed oil. We incubated crude microsomal fractions from developing seeds with PC having ^{14}C-labeled oleoyl, ricinoleoyl or vernoloyl groups at the *sn*-2 position. After the incubation, lipids were extracted and analyzed by thin layer chromatography. We found that the amount of radioactivity that was incorporated into the neutral lipid fraction increased linearly over a period of 4 hours (data not shown). The distribution of [^{14}C]acyl groups within the neutral lipid fraction was analyzed after 80 min (Fig. 1). Interestingly the amount and distribution of radioactivity between diffferent neutral lipids were strongly dependent both on the plant species and on the type of [^{14}C]acyl chain. Thus, sunflower microsomes incorporated most of the label into DAG, regardless of the type of [^{14}C]acyl group. In contrast, *R. communis* microsomes preferentially incorporated [^{14}C]ricinoleoyl and [^{14}C]vernoloyl groups

into TAG, while [^{14}C]oleyl groups mostly were found in DAG. *C. palaestina* microsomes, finally, incorporated only [^{14}C]vernolyol groups into TAG, with [^{14}C]ricinoleyl groups being found mostly as free fatty acids, and [^{14}C]oleyl groups in DAG. From these studies we conclude that the high *in vivo* levels of ricinoleic acid and vernolic acid in the TAG pool of *R. communis* and *C. palaestina*, respectively, may explained by an efficient and selective transfer of the corresponding acyl groups from PC to TAG in these organisms.

PDAT: a novel enzyme that catalyzes acyl-CoA independent synthesis of TAG. It has previously been suggested that an acyl-CoA-independent synthesis of TAG from PC could be catalyzed by a DAG : DAG acyltransferase (7,8), since it is known that acyl chains can be transferred from PC to DAG by the reversed action of a CDP-choline : choline phosphotransferase (24). We therefore investigated if DAG could serve both as an acyl donor as well as an acyl acceptor in the reactions catalyzed by the oil seed microsomes. To this end, we incubated unlabeled divernoloyl-DAG with either *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG or *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC in the presence of *R. communis* microsomes. We found that the synthesis of TAG molecules containing both [^{14}C]ricinoleoyl and vernoloyl groups was 5 fold higher when [^{14}C]ricinoleoyl-PC served as acyl donor as compared to [^{14}C]ricinoleoyl-DAG (fig.1B). These data strongly suggests that PC is the immediate acyl donor and DAG the acyl acceptor in the acyl-CoA-independent formation of TAG by oil seed microsomes. We therefore propose that this reaction is catalyzed by a new enzyme which we call phospholipid : diacylglycerol acyltransferase (PDAT).

PDAT activity is also present in yeast microsomes. In an attempt to identify the gene(s) coding for this new enzyme, we considered the possibility that it might be present in other oil accumulating organisms. It is well known that yeast cells can accumulate substantial amount of TAG especially during the late exponential and stationary phase, or following exhaustion of certain growth medium components (25). Therefore, we investigated if budding yeast (*Saccharomyces cerevisiae*) contains a PDAT activity that can contribute to TAG synthesis *in vivo*. Wild type yeast cells were cultivated under conditions where TAG synthesis is induced. Microsomal membranes were prepared from these cells and incubated with *sn*-2-[^{14}C]-ricinoleoyl-PC and DAG and the ^{14}C-labeled products formed were analyzed. We found that the PC-derived [^{14}C]ricinoleoyl groups within the neutral lipid fraction mainly were found

in free fatty acids or TAG, and also that the amount of TAG synthesized was dependent on the amount of DAG that was added to the reaction (Fig.2). The *in vitro* synthesis of TAG containing both ricinoleoyl and vernoloyl groups, a TAG species not present *in vivo*, from exogenous added *sn*-2-[^{14}C]ricinoleoyl-PC and unlabilled vernoloyl-DAG (Fig. 2, lane 3) clearly demonstrates the existence of an acyl-CoA-independent synthesis of TAG involving PC and DAG as substrates in yeast microsomal membranes. We conclude from these experiments that TAG synthesis in yeast can be catalyzed by an enzyme similar to the PDAT found in plants.

Identification of the PDAT encoding gene in yeast. In mammalian cells, the synthesis of cholesterol esters is mainly catalyzed by the enzyme acyl-CoA : cholesterol acyltransferases (ACAT), which is related to DAGAT. However, the synthesis of cholesterol esters can also occur in an acyl-CoA independent reaction where an acyl group is transferred from PC to cholesterol. This reaction is catalyzed by lecithin: cholesterol acyltransferase (LCAT), an enzyme that is found in mammalian blood (26) The sequence homologies between LCAT and DAGAT enzymes, using cholesterol and diacylglycerols as acyl acceptors, raised the possibility that an LCAT-related enzyme could be responsible for the PDAT activity found in yeast and plant microsomes.

A homology search revealed that there is one gene in the yeast genome, YNR008w, that show a significant sequence similarity to LCAT. We therefore considered the possibility that YNR008w might encode the PDAT activity. Nothing is known about the function of YNR008w, except that the gene is not essential for growth under normal circumstances. We obtained a strain from the Euroscarf collection, FVKT004-04C(AL) (9) in which this gene had been disrupted and found that the disruption has no apparent effect on the sensitivity to temperature, salt or oxidative stress, or nitrogen starvation, nor does it affect growth on different carbon sources. To test if YNR008w might encode a PDAT enzyme, we prepared microsomal membranes from this strain and assayed for PDAT activity using PC labeled at the sn-2 position with radioactive fatty acids. The activity was found to be completely absent in the disruption strain (Fig. 2 lane 4). Significantly, the activity could be partially restored by the presence of YNR008w on the single copy plasmid pUS1 (Fig. 2 lane 5). Moreover, we found that also acyl groups of phosphatidylethanolamine (PE) were efficiently incorporated into TAG by microsomes from the wild type strain whereas no

incorporation occured from this substrate in the mutant strain (data not shown). Taken together, these findings show that YNR008w encodes a yeast PDAT which catalyzes the transfer of an acyl group from the *sn*-2 position of phospholipids to DAG, thus forming TAG. It should be noted that no cholesterol esters were formed from radioactive PC even in incubations with added ergosterols, nor were the amount of radioactive free fatty acids formed from PC affected by disruption of the YNR008w gene (data not shown). This demonstrates that yeast PDAT do not have cholesterol ester synthesising or phospholipase activities.

The expression of the PDAT gene in wild type yeast was analyzed by Northern blots with RNA isolated from yeast cells grown in rich medium and harvested at different time points ranging from mid exponential to late stationary phase (data not shown). We found that the level of expression was always low, but slightly higher during exponential growth as compared to cells in the stationary phase, where the mRNA was barely detectable. Thus, the expression of the PDAT gene does not coincide with the major stage for TAG accumulation, which occurs during the late stationary phase in yeast.

Lipid analyses were performed on both wild type and mutant yeast cells in order to determine the effect of the YNR008w disruption on the lipid composition. We found that the mutant as compared to the wild type strain only showed minor differences in the amounts of major polar lipids (PC, PE and phosphatidylinositol), sterolesters and triacylglycerols, regardless of whether the cells were harvested in exponential or stationary phase (data not shown). From these experiments we conclude that while the PDAT activity encoded by YNR008w may contribute to TAG synthesis *in vivo*, other pathways are able to fully compensate for the loss of PDAT activity in the mutant strain. The data also suggests that the PDAT enzyme is not be the major contributor to the high levels of TAG accumulated in yeast at late stationary phase.

Increased TAG content in yeast cells that overexpress PDAT. The effect of overexpressing the PDAT-encoding gene was studied by transforming a wild type yeast strain with the pUS4 plasmid in which the gene is expressed from the galactose-induced *GAL1:TPK2* promoter. As a control cells containing the empty expression vector were used. The cells were grown in synthetic glycerol-ethanol medium, and expression of the gene was induced after either 2 hours (early log phase) or 25 hours

(stationary phase) by the addition of galactose. The cells were then incubated for another 21 hours, after which they were harvested and assays were performed. We found that overexpression of PDAT had no significant effect on the growth rate as determined by the optical density. However, the total lipid content, measured as total μmol fatty acids per mg yeast dry weight, was 47% (log phase) or 29% (stationary phase) higher in the PDAT overexpressing strain than in the control. Furthermore, we found that the polar lipid and sterolester content was unaffected by overexpression of PDAT. Instead, the elevated lipid content in these cells is entirely due to an increased TAG content (Fig. 3A,B). Thus, the amount of TAG was increased by 2-fold in PDAT overexpressing early log phase cells and by 40% in stationary phase cells. It is interesting to note that a significant increase in the TAG content was achieved by overexpressing PDAT even under conditions (*i.e.* in stationary phase) where DAGAT is induced and thus contributes significantly to TAG synthesis. *In vitro* PDAT activity assayed in microsomes from the PDAT overexpressing strain was 7-fold higher than in the control strain, a finding which is consistent with the increased levels of TAG that we observed *in vivo* (Fig. 3C). These results clearly demonstrate the potential use of the PDAT gene in increasing the oil content in transgenic organisms.

Substrate specificity of yeast PDAT. The enzymatic activity of LCAT has been thoroughly characterized and shown to have a preference for transfer of the *sn*-2 acyl group of PC (27). The substrate specificity of yeast PDAT was analyzed using microsomes prepared from the PDAT overexpressing strain (see Fig. 4). We found that the rate of TAG synthesis, under conditions given in figure 4 with di-oleoyl-PC as the acyl-donor, was 0.15 nmol per min and mg protein. With both oleoyl groups of PC labeled we were able, under the given assay conditions, to detect the transfer of 11 pmol/min of [^{14}C]oleoyl chain into TAG and the formation of 15 pmol/min of lyso-PC. In microsomes from the PDAT-deficient strain, no TAG at all and only trace amounts of lyso-PC was detected, strongly suggesting that yeast PDAT catalyses the formation of equimolar amounts of TAG and lyso-PC when supplied with PC and DAG as substrates. The fact that somewhat more lyso-PC than TAG is formed can be explained by the presence of a phospholipase in yeast microsomes, which produces lyso-PC and unesterified fatty acids from PC (data not shown).

The specificity of yeast PDAT for different acyl group positions was investigated by incubating the microsomes with di-oleoyl-PC carrying a [^{14}C]acyl

group either at the *sn*-1 position (Fig. 4A bar 2) or the *sn*-2 position (Fig. 4A bar 3). We found that the major ^{14}C-labeled product formed in the former case was lyso-PC, and in the latter case TAG. We conclude that yeast PDAT has a specificity for the transfer of acyl groups from the *sn*-2 position of the phospholipid to DAG, thus forming *sn*-1-lyso-PC and TAG. Under the given assay conditions, trace amounts of ^{14}C-labelled DAG is formed from the *sn*-1 labeled PC by the reversible action of a CDP-choline : choline phosphotransferase (data not shown). This labeled DAG can then be further converted into TAG by the PDAT activity. It is therefore not possible to distinguish whether the minor amounts of labeled TAG that is formed in the presence of di-oleoyl-PC carrying a [^{14}C]acyl group in the *sn*-1 position, is synthesized directly from the *sn*-1-labeled PC by a PDAT that also can act on the *sn*-1 postion, or if it is first converted to *sn*-1-labeled DAG and then acylated by a PDAT with strict selectivity for the transfer of acyl groups at the *sn*-2 position of PC. Taken together, our experiments suggest that the PDAT encoded by YNR008w catalyses an acyl transfer from the *sn*-2 position of PC to DAG, thus causing the formation of TAG and lyso-PC.

The substrate specificity of yeast PDAT was further analyzed with respect to the headgroup of the acyl donor, the acyl group transferred and the acyl chains of the acceptor DAG molecule. The two major membrane lipids of *S. cerevisiae* are PC and PE, and as shown in Fig. 4B (bars 1 and 2), dioleoyl-PE is nearly 4-fold more efficient than dioleoyl-PC as acyl donor in the PDAT-catalyzed reaction. Moreover, the rate of acyl transfer is strongly dependent on the type of acyl group that is transferred. Thus, a ricinoleoyl group at the *sn*-2 position of PC is 2.5 times more efficiently transferred into TAG than an oleoyl group in the same position (Fig. 4B bars 1 and 3). In contrast, yeast PDAT has no preference for the transfer of vernoloyl groups over oleoyl groups (Fig. 4B bars 1 and 4). The acyl chain of the acceptor DAG molecule also affects the efficiency of the reaction. Thus, DAG with a ricinoleoyl or a vernoloyl group is a more efficient acyl acceptor than dioleoyl-DAG (Fig. 4B bars 1, 5 and 6). Taken together, these results clearly show that the efficiency of the PDAT-catalyzed acyl transfer is strongly dependent on the properties of the substrate lipids.

Three PDAT-related proteins form a separate branch within the LCAT protein superfamily. In order to identify possible PDAT homologs in other organisms, we searched the data bases for related sequences. A large number of

protein sequences from different eukaryotes show similarity to both LCAT and PDAT. To further analyze how they are related to each other, we used the most conserved part of the sequences, where all proteins can be unambiguously aligned, to compute a dendrogram (Fig. 5). This conserved region comprises the N-terminal third of the proteins and includes the putative lipase active site motif, HS(M/L)G. Interestingly, while most of the available sequences show a strong similarity to human and mouse LCAT, two sequences (one from fission yeast and one from *A. thaliana*) are clearly more closely related to the budding yeast PDAT than to any of the LCATs. It is therefore likely that these two enzymes also possess PDAT activity. The presence of PDAT-related proteins in both fungi and plants suggests that a distinct branch encoding enzymes with this activity arose at an early point in evolution.

DISCUSSION

During the past few years, the biosynthesis of TAG has received much attention due to its central role both in fat accumulation in animal cells and in the synthesis of storage lipids in oil seed plants. Until recently, the only enzyme thought to be directly involved in the synthesis of TAG was DAGAT, which catalyses the acyl-CoA-dependent acylation of DAG into TAG. Genes encoding enzymes with DAGAT activity have recently been identified in the mouse (3), in plants (4, 5) and in microbes (6). We have now shown that plant and yeast cells also can synthesize TAG by a novel reaction where a phospholipid acts as the acyl donor. This reaction is catalyzed by an acyl-CoA-independent enzyme which we call phospholipid: diacylglycerol acyltransferase or PDAT.

It is clear from the results in Fig. 1 that the specificity of the PDAT enzyme with respect to the acyl group that is transferred from PC into TAG varies between different plant species. Thus, either the ricinoleoyl or the vernoloyl group of PC is specifically incorporated into TAG when microsomal fractions from Castor bean and *C. palaestina* are used, respectively. This suggests that PDAT could play an important role in the specific channeling of bilayer disturbing fatty acids (*e. g.* ricinoleic and vernolic acid) from PC into the triacylglycerol pool. The synthesis of ricinoleic and vernolic acid in transgenic *A. thaliana* has been reported (22, 23, 30), but only low to

moderate levels of these fatty acids were observed in the seed oil. It is conceivable that that the expression of a PDAT enzyme with specificity for the desired unusual fatty acid could help to achieve higher levels of ricinoleic and vernolic acids in the seed oil of these transgenic plants.

The physiological role of PDAT and its intracellular location remain to be determined. However, it should be noted that budding yeast PDAT and its homologs in fission yeast and *Arabidopsis* all share an N-terminal extension of 80-140 amino acid residues which is absent in the other LCAT-related enzymes. Moreover, this extension contains a predicted membrane-spanning region, which could serve to anchor the enzyme in a membrane. As for the *in vivo* function of PDAT, it is clear from our results that the *in vivo* accumulation of TAG in yeast is not dependent on PDAT. This suggests that the CoA-dependent synthesis of TAG, which is catalyzed by DAGAT, is sufficient to maintain adequate TAG levels under normal conditions. It is therefore conceivable that the yeast PDAT may have some other more specialized role *in vivo*. In this context, we note that PDAT also catalyzes a breakdown of the major membrane lipids (PC and PE). Possibly, yeast PDAT could be involved in the regulation of membrane lipid composition in response to different growth conditions. As shown in Fig. 4B, the two major membrane lipids in yeast, PC and PE, can both act as acyl donors in the synthesis of TAG by PDAT. However, the rate of synthesis is clearly dependent on the polar head group, the type of acyl group that is transferred and the acyl groups of the acceptor DAG molecule. Taken together, these results suggest that the substrate specificity of PDAT may play an important role in determining the membrane lipid composition *in vivo*.

The dendrogram in Fig. 5 suggests that separate branches leading to the PDAT-related and LCAT-related enzymes arose at an early stage in eukaryotic evolution. Interestingly, while PDAT is the only member of the LCAT protein family which has so far been found in fungi, the opposite is true in animals, where only LCAT-related but no PDAT-related enzymes have been found. This raises the question whether the separation of PDAT-related and LCAT-related enzymes in fact reflects not an ancient gene duplication but rather the divergence of fungi from animals. According to this view, PDAT would be the true homolog of LCAT in fungi, though with a substrate specificity that differs considerably from the latter. However, an examination of the available plant sequences strongly suggests that this is not the case. Thus, *A. thaliana*

has one clearly PDAT-related and one LCAT-related sequence, as well as two other more divergent members of the same protein superfamily, one of which is closer to PDAT than to LCAT within the dendrogram (Fig. 5). We conclude that separate enzymes encoding PDAT and LCAT most likely were present already in the common ancestor of plant, animals and fungi, and that the apparent absence of LCAT in fungi and PDAT in animals probably reflects a more recent loss of these enzymes in the respective kingdoms.

In conclusion, we have found a new enzyme that we call phospholipid : diacylglycerol acyltransferase or PDAT, and which is present in both plants and yeast. It catalyzes the transfer of acyl groups from the *sn*-2 position of the major phospholipids to DAGs, thus forming TAGs and lyso-phospholipids. The yeast gene encoding the PDAT enzyme was identified, and the encoded protein was found to be related to the human enzyme LCAT. The activity of yeast PDAT was shown to be dependent on the type of polar head-group of the donor lipid, the acyl group transferred, and on the acyl chains of the acceptor molecule DAG. While the *in vivo* function of PDAT still remains to be determined, it is conceivable that it could play a role in regulating the fatty acid composition of membrane lipids.

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REFERENCES

1. Bell, R. M. & Coleman, R. A. (1980) *Annu. Rev. Biochem.* **49**, 459-487.
2. Stymne, S. & Stobart, K. (1987) in *The biochemistry of plants: a comprehensive treatsie, Vol. 9*, ed. Stumpf, P. K. (Academic Press, New York), pp. 175-214.
3. Cases, S. et al. (1998) *Proc. Natl. Acad. Sci. U S A* **95**, 13018-13023.
4. Hobbs, D. H., Lu, C. & Hills, M. J. (1999) *FEBS Lett.* **452**, 145-9
5. Zou, J., Wei, Y., Jako, C., Kumar, A., Selvaraj, G. & Taylor, D. C. (1999) *Plant J.* **19**, 645-653.
6. Lardizabal, K., Hawkins, D., Mai, J., & Wagner, N. (1999) Abstract presented at the Biochem. Mol. Biol. Plant Fatty Acids Glycerolipids Symposium, South Lake Tahoe, USA.
7. Stobart, K., Mancha, M. & Lenman M, Dahlqvist, A. & Stymne, S. (1997) *Planta* **203**, 58-66.
8. Dahlqvist, A. & Stymne, S. (1998) in *Advances in Plant Lipid Research*, eds. Sánches, J., Cerdá-Olmedo, E. & Martinez-Force, E. (Universidad de Sevilla), pp. 211-214.
9. Entian, K.-D. & Kötter, P. (1998) *Meth. Microbiol.* **26**, 431-449.
10. Thomas, B. J. & Rothstein, R. (1989) *Cell* **56**, 619-630.
11. Kern, L., de Montigny, J., Jund, R. & Lacroute, F. (1990) *Gene* **88**, 149-157.
12. Ronne, H., Carlberg, M., Hu, G.-Z. & Nehlin, J. O. (1991) *Mol. Cell. Biol.* **11**, 4876-4884.
13. Stobart, K. & Stymne, S. (1990) in *Method in Plant Biochemistry, vol 4,* eds. Harwood, J. L. & Bowyer, J. R. (Academic press, London), pp. 19-46.
14. Bafor, M., Smith, M. A., Jonsson, L., Stobrt, A. K. & Stymne, S. (1991) *Biochem. J.* **280**, 507-514.
15. Banas, A., Johansson, I. & Stymne, S. (1992) *Plant Science* **84**, 137-144.
16. Kanda, P. & Wells, M. A. (1981) *J. Lipid. Res.* **22**, 877-879.
17. Ståhl, U., Ek, B. & Stymne, S. (1998) *Plant Physiol.* **117**, 197-205.
18. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911-917.
19. Sherman, F., Fink, G. R. & Hicks, J. B. (1986) in *Laboratory Course Manual for Methods in Yeast Genentics* (Cold Spring Harbor Laboratory)

20. Meesters, P. A. E. P., Huijberts, G. N. M. and Eggink, G. (1996) *Appl. Microbiol. Biotechnol.* **45**, 575-579.

21. van de Loo, F. J., Fox, B. G. & Sommerville, C. (1993), in *Lipid metabolism in plants*, ed. Moore, T. S. (CRC Press, Inc.), pp. 91-126.

22. van de Loo, F. J., Broun, P., Turner, S. & Sommerville, S. (1995) *Proc. Natl. Acad. Sci. USA* **95**, 6743-6747.

23. Lee, M. et al. (1998) *Science* **280**, 915-918.

24. Slack, C. R., Roughan, P. G., Browse, J. A. & Gardiner, S. E. (1985) *Biochim. Biophys. Acta* **833**, 438-448.

25. Rattray, J. B., Schibeci, A., and Kidby, D. K. (1975) *Bacteriol. Rev.* **39**, 197-231.

26. Glomset, J. A. (1968) *J. Lipid Res.* **9**, 155-167.

27. Veedamali, M. L., Subramanian, S. & Subbaiah, P. V. (1998) *Biochemistry* **37**, 13626-13633.

28. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) *Nucl. Acids Res.* **24**, 4876-4882.

29. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406-425.

30. Broun, P., Boddupalli, S. & Somerville, C. (1998) *Plant J.* **13**, 201-210.

FIGURE LEGENDS

FIG. 1. Metabolism of ^{14}C-labeled PC into the neutral lipid fraction by plant microsomes. (A) Microsomes from developing seeds of sunflower, *R. communis* and *C. palaestina* were incubated for 80 min at 30°C with PC (8 nmol) having oleic acid in its *sn*-1 position, and either ^{14}C-labeled oleic, ricinoleic or vernolic acid in its *sn*-2 position. Radioactivity incorporated in TAG (open bars), DAG (solid bars), and unsterified fatty acids (hatched bars) was quantified using thin layer chromatography followed by electronic autoradiography, and is shown as percentage of added labeled substrate. (B) Synthesis *in vitro* of TAG carrying two vernoloyl and one [^{14}C]ricinoleoyl group by microsomes from *R. communis*. The substrates added were unlabeled divernoloyl-DAG (5 nmol), together with either *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG (0.4 nmol, 7700 dpm/nmol) or *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC (0.4 nmol, 7700 dpm/nmol). The microsomes were incubated with the substrates for 30 min at 30°C, after which samples were removed for lipid analysis as described in Material and Methods. The data shown are the average of two experiments.

FIG. 2. PDAT activity in yeast microsomes. Microsomal membranes (10 nmol of PC) from the wild type yeast strain FY1679 (lanes 1-3), a congenic yeast strain (FVKT004-04C(AL)) that is disrupted for YNR008w (lane 4) or the same disruption strain transformed with the plasmid pUS1, containing the YNR008w gene behind its native promotor (lane 5), were assayed for PDAT activity. As substrates, we used 2 nmol *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC together with either 5 nmol of dioleoyl-DAG (lanes 2, 4 and 5) or *rac*-oleoyl-vernoleoyl-DAG (lane 3). The enzymatic assay and lipid analysis was performed as described in Materials and Methods. The cells were precultured for 20 h in liquid YPD medium, harvested and re-suspended in an equal volume of minimal medium (Meesters et al, 1996) containing 16 g/l glycerol. The cells were then grown for an additional 24 h prior to being harvested. Selection for the plasmid was maintained by growing the transformed cells in synthetic medium lacking uracil (Sherman et al. 1986). Abbreviations: 1-OH-TAG, monoricinoleoyl-TAG; 1-OH-1-ep-TAG, monoricinoleoyl-monovernoloyl-TAG; OH-FA, unesterified ricinoleic acid.

Fig. 3. Lipid content (A,B) and PDAT activity (C) in PDAT overexpressing yeast cells. The PDAT gene in the plasmid pUS4 was overexpressed from the galactose-induced *GAL1-TPK2* promotor in the wild type strain W303-1A (10). Its expression was induced after (A) 2 hours or (B) 25 hours of growth by the addition of 2% final concentration (w/v) of galactose. The cells were then incubated for another 22 hours before being harvested.. The amount of lipids of the harvested cells was determined by GLC-analysis of its fatty acid contents and is presented as μmol fatty acids per mg dry weight in either TAG (open bar), polar lipids (hatched bar), sterol esters (solid bar) and other lipids (striped bar). The data shown are the mean values of results with three independent yeast cultures. (C) *In vitro* synthesis of TAG by microsomes prepared from yeast cells containing either the empty vector (vector) or the PDAT plasmid (+ PDAT). The cells were grown as in Fig. 3A. The substrate lipids dioleoyl-DAG (2.5 nmol) and *sn*-1-oleoyl-*sn*-2-[^{14}C]-oleoyl-PC (2 nmol) were added to aliquots of microsomes (10 nmol PC), which were then incubated for 10 min at 28 °C. The amount of label incorporated into TAG was quantified by electronic autoradiography. The results shown are the mean values of two experiments.

FIG. 4. Substrate specificity of yeast PDAT. The PDAT activity was assayed by incubating aliquots of lyophilized microsomes (10 nmol PC) with substrate lipids at 30°C for 10 min (panel A) or 90 min (panel B). Unlabeled DAG (2.5 nmol) was used as substrates together with different labeled phospholipids, as shown in the figure. (A) *Sn*-position specificity of yeast PDAT regarding the acyl donor substrate. Dioleoyl-DAG together with either *sn*-1-[^{14}C]oleoyl-*sn*-2-[^{14}C]oleoyl-PC (di-[^{14}C]-PC), *sn*-1-[^{14}C]oleoyl-*sn*-2-oleoyl-PC (*sn*1-[^{14}C]-PC) or *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PC (*sn*2-[^{14}C]-PC). (B) Specificity of yeast PDAT regarding phospholipid headgroup and of the acyl composition of the phospholipid as well as of the diacylglycerol. Dioleoyl-DAG together with either *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PC (oleoyl-PC), *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PE (oleoyl-PE), *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC (ricinoleoyl-PC) or *sn*-1-oleoyl-*sn*-2-[^{14}C]vernoloyl-PC (vernoloyl-PC). In the experiments presnted in the 2 bars to the far right, monoricinoleoyl-DAG (ricinoleoyl-DAG or mono-vernoloyl-DAG (vernoloyl-DAG) were used together with *sn*-1-oleoyl-*sn*-2-[^{14}C]-oleoyl-PC. The label that was incorporated into TAG (solid bars) and lyso-PC

(LPC, open bars) was quantified by electronic autoradiography. The results shown are the mean values of two experiments. The microsomes used were from W303-1A cells overexpressing the PDAT gene from the *GAL1-TPK2* promotor, as described in Fig. 3. The expression was induced at early stationary phase and the cells were harvested after an additional 24 h.

FIG. 5. Evolutionary dendrogram showing LCAT and PDAT related proteins from different eukaryotes. The dendrogram was calculated from aligned protein sequences corresponding to amino acid residues 174-335 in yeast PDAT. The ClustalX program (28) was used with default settings to compute pairwise alignment scores. An unrooted tree was then obtained using the Neighbour-Joining method (29), with correction for multiple substitutions and exclusion of gapped positions.

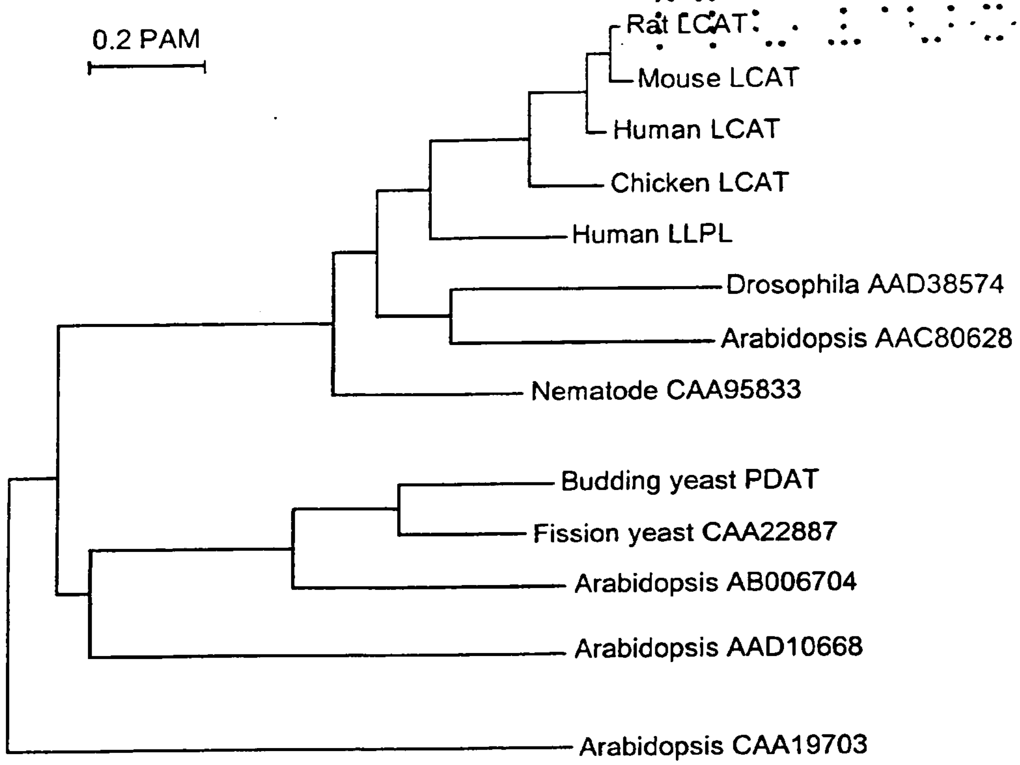

0.2 PAM
Rat LCAT
Mouse LCAT
Human LCAT
Chicken LCAT
Human LLPL
Drosophila AAD38574
Arabidopsis AAC80628
Nematode CAA95833
Budding yeast PDAT
Fission yeast CAA22887
Arabidopsis AB006704
Arabidopsis AAD10668
Arabidopsis CAA19703

FIGURE 1

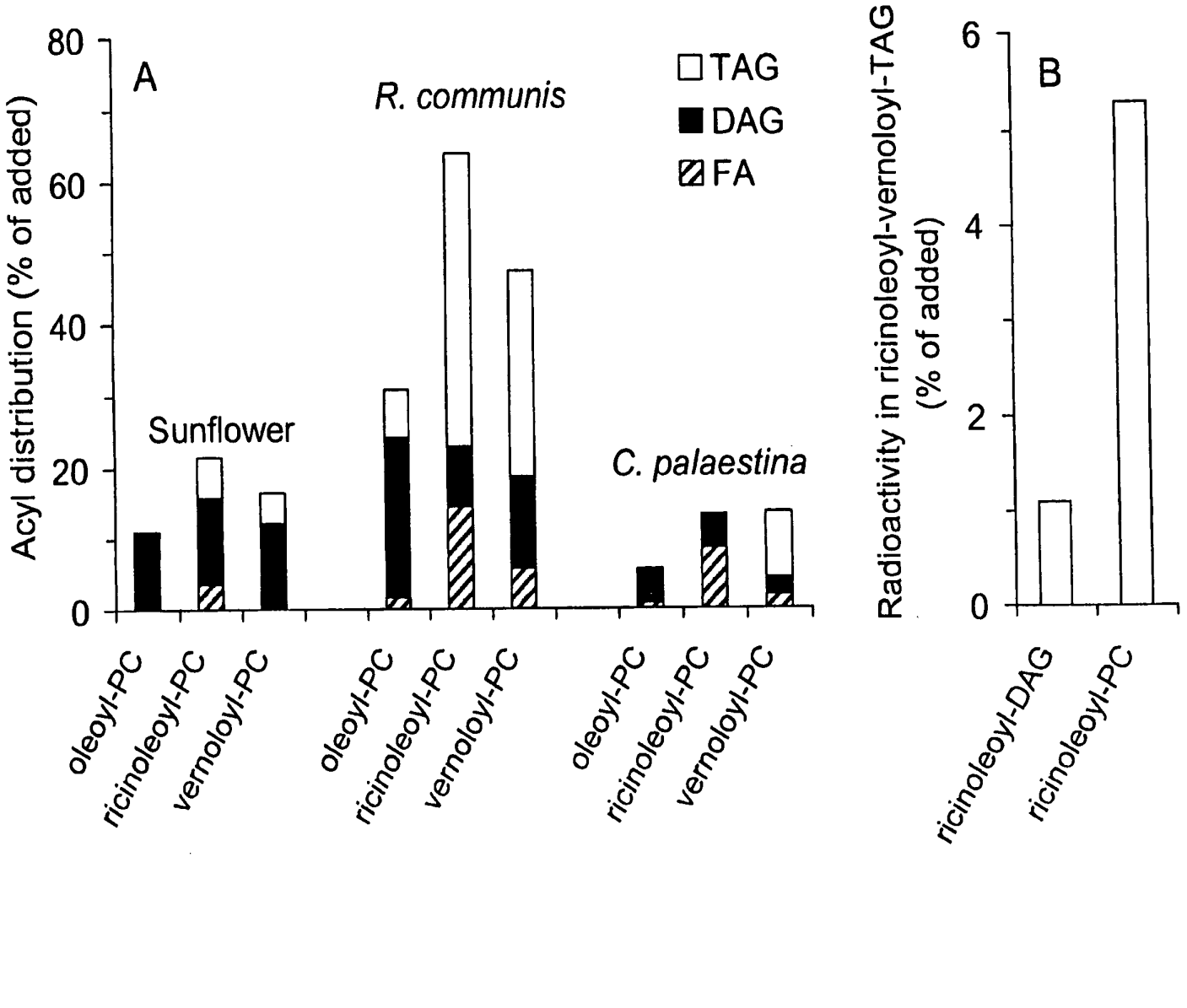

A
R. communis
TAG
DAG
FA
Acyl distribution (% of added)
80
60
40
20
0
Sunflower
C. palaestina
oleoyl-PC
ricinoleoyl-PC
vernoloyl-PC
oleoyl-PC
ricinoleoyl-PC
vernoloyl-PC
oleoyl-PC
ricinoleoyl-PC
vernoloyl-PC
B
Radioactivity in ricinoleoyl-vernoloyl-TAG (% of added)
6
4
2
0
ricinoleoyl-DAG
ricinoleoyl-PC

Fig 2

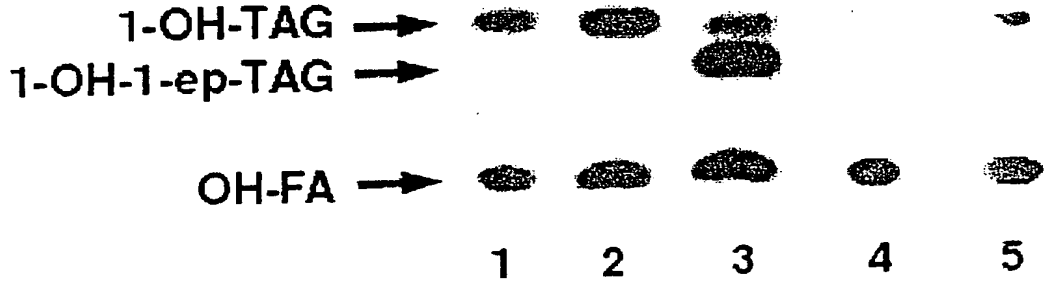


FIGURE 4

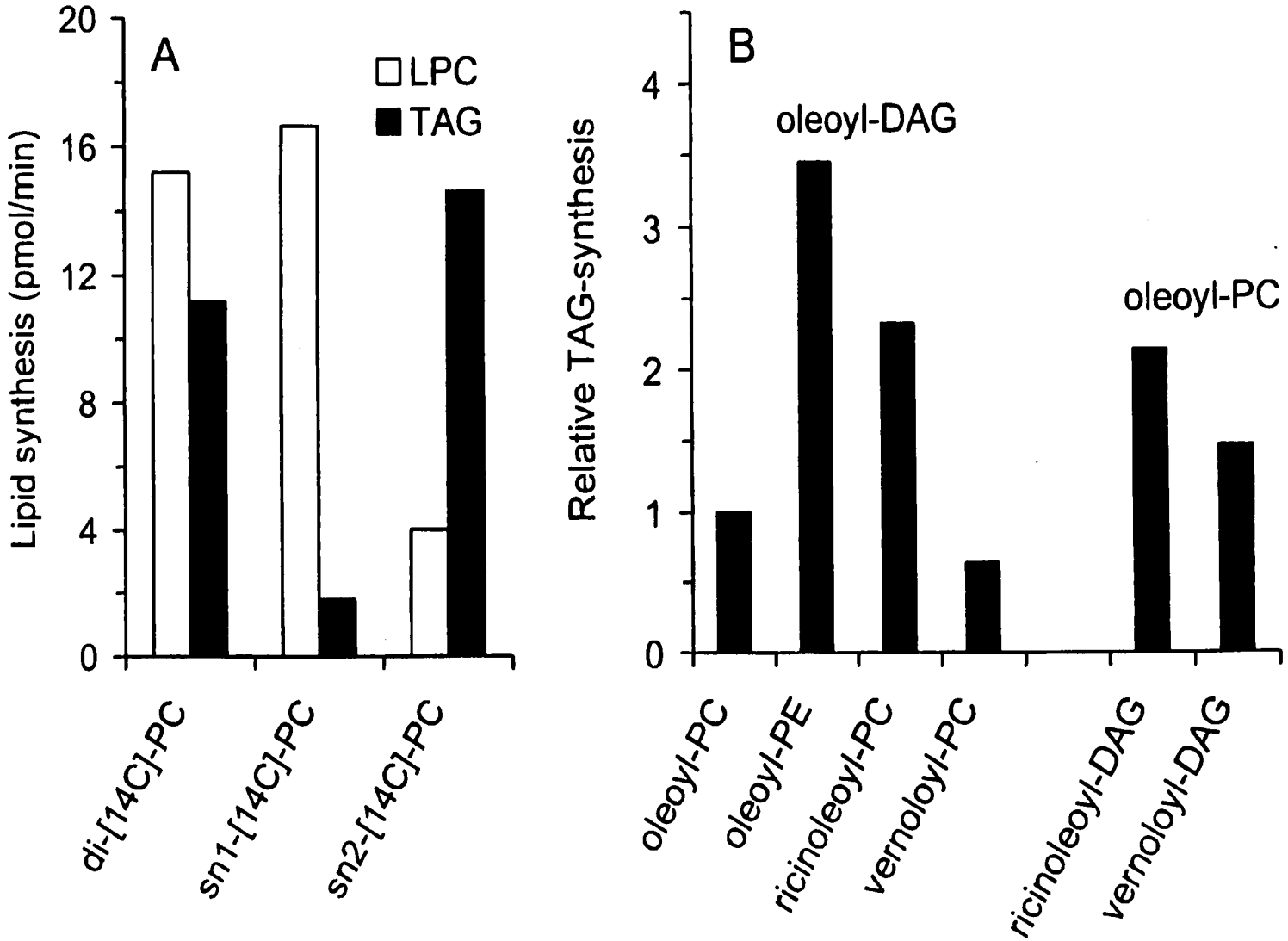
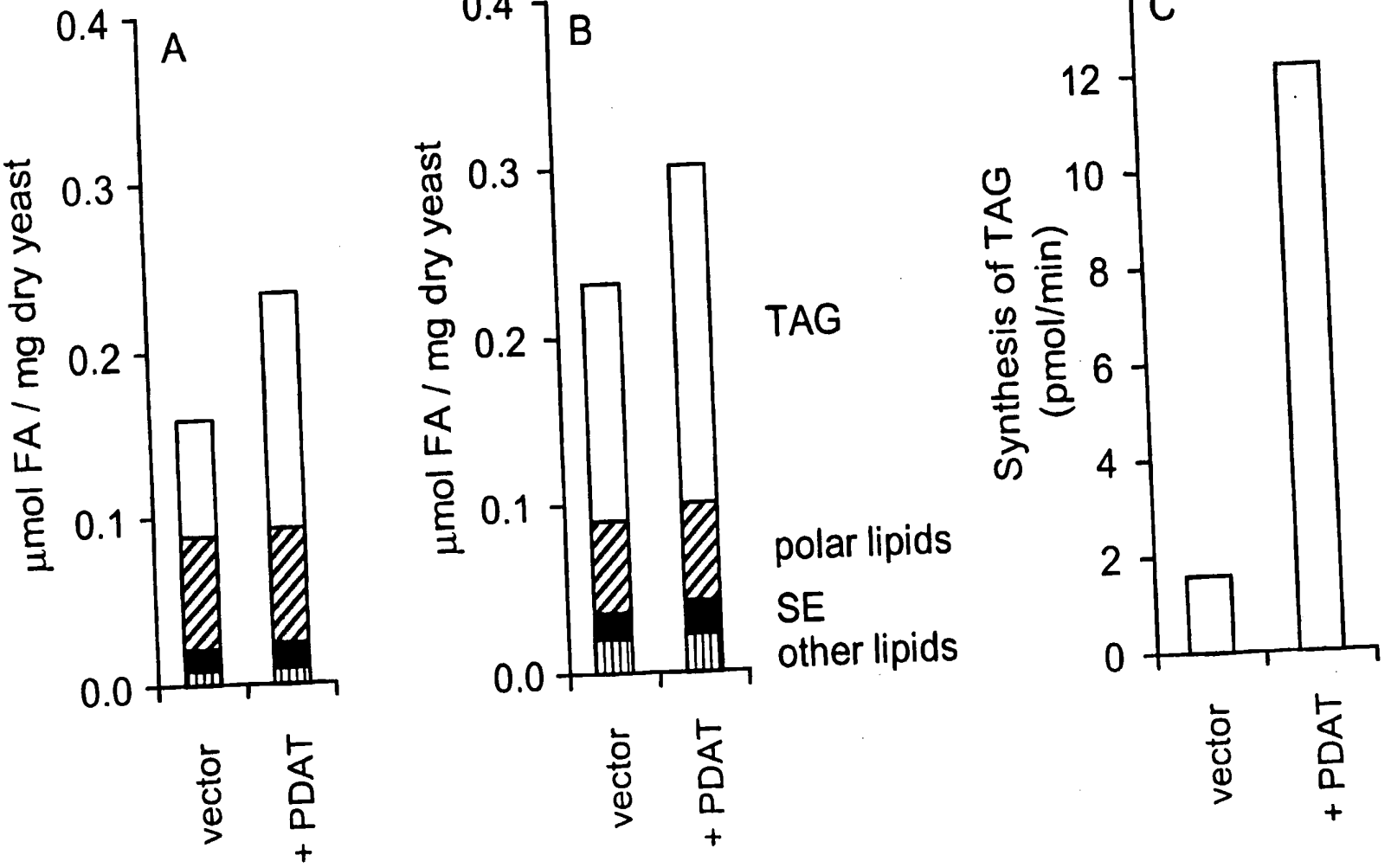

A
LPC
TAG
Lipid synthesis (pmol/min)
20
16
12
8
4
0
di-[14C]-PC
sn1-[14C]-PC
sn2-[14C]-PC
B
Relative TAG-synthesis
4
3
2
1
0
oleoyl-DAG
oleoyl-PC
oleoyl-PC
oleoyl-PE
ricinoleoyl-PC
vernoloyl-PC
ricinoleoyl-DAG
vernoloyl-DAG

FIGURE 3


A
B
C
µmol FA / mg dry yeast
Synthesis of TAG (pmol/min)
0.4
0.3
0.2
0.1
0.0
14
12
10
8
6
4
2
0
vector
+ PDAT
TAG
polar lipids
SE
other lipids